IMPAIRED HEMORHEOLOGICAL PARAMETERS AND RESISTANCE TO ROUTINE ANTIPLATELET THERAPY

Ph.D. thesis

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LIST OF ABBREVIATIONS (in the order of appearence)

Ischemic heart disease	(IHD)
Body mass index	(BMI)
Arterial hypertension	(AHT)
Thromboxane A2	(TXA_2)
Cyclo-oxygenase	(COX)
Prostaglandin E	(PGE)
Endothelium-derived prostacyclin	(PGI2)
Messenger ribonucleic acid	(mRNA)
Platelet endothelial cell adhesion molecule-1	(PECAM-1)
Adenosine diphosphate	(ADP)
Serine residue at position 529	(Ser529)
Acetylsalicylic acid/aspirin	(ASA)
Clopidogrel	(CLP)
Coronary artery disease	(CAD)
Acute myocardial infarction	(AMI)
Transient ischemic attack	(TIA)
Angiotensine converting enzyme	(ACE)
Angiotensine II	(AT II)
Calcium	(CA)
Proton pump inhibitor	(PPI)
Trimetazidine	(TMZ)
Selective serotonin reuptake inhibitor	(SSRI)
Red blood cell	(RBC)
Cardiovascular disease	(CVD)
Tissue plasminogen activator	(t-PA)
C-reactive protein	(CRP)
Platelet rich plasma	(PRP)
Platelet poor plasma	(PPP)
Standard deviation	(SD)
Nonsteroidal anti-inflammatory drug	(NSAID)
von Willebrand factor	(vWf)
Soluble P-selectin	(sP-selectin)
Cytochrome P 450	(CYP)
Arachidonic acid	(AA)
Percutaneous coronary intervention	(PCI)
Vasodilator-stimulated phosphoprotein	(VASP)

1. INTRODUCTION

1.1 Hemorheological parameters in atherothrombosis

Cardio- and cerebrovascular diseases and their complications are the leading cause of morbidity and mortality in the developed world. In the development of these diseases several factors are involved that interact with each other. While the role of "classic" risk factors (e.g., smoking, hypertension, hypercholesterolemia, diabetes mellitus, etc.) has been well known for a long time, the importance of hemorheologic parameters (hematocrit, fibrinogen, red blood cell deformability, red blood cell aggregation, plasma and whole blood viscosities) in the development of arteriosclerotic disorders has been accepted only in the last two decades. It is generally agreed that a rise in hemorheological factors leads to a state of hypoperfusion which results in impaired microcirculation (1-3).

Hemorheology is concerned with the flow properties of cellular and plasmatic components of blood. The resistance of blood to flow is known as blood viscosity. High blood viscosity slows down the blood flow and results in stasis and occlusion. Blood viscosity is determined by hematocrit, plasma viscosity, erythrocyte aggregation and cellular deformability. Since erythrocytes make up approximately 99% of the blood cells, erythrocyte deformability is an important parameter (4,5).

Normal erythrocytes are significantly deformable. This property enables them to adapt well to flow conditions throughout the circulation. Due to the deformability of erythrocytes, blood viscosity is lower than the viscosity of a fluid containing non-deformable particles of the same size. When erythrocytes become less deformable, the viscosity of blood increases. The deformability of an erythrocyte is determined by three factors: viscoelasticity of its membrane which is controlled by a network of cytoskeletal proteins, biconcave-disk cell shape with a large ratio of surface area/cell volume, and intracellular viscosity which is mainly determined by hemoglobin concentration (5).

The viscosity of plasma depends on its macromolecular content, the plasma proteins in particular. Among the plasma proteins, fibrinogen is the one that most affects plasma viscosity. Although fibrinogen constitutes a very small portion of the plasma proteins, its thin and long shape makes it an important determinant. Simple measurement of fibrinogen level thus may give an idea about plasma viscosity. However, protein–protein interactions also have important effects on plasma viscosity, especially during pathological conditions.

Determination of plasma viscosity by a viscometer not only reflects the effect of fibrinogen but that of protein–protein interactions as well (6) (see Figure1).

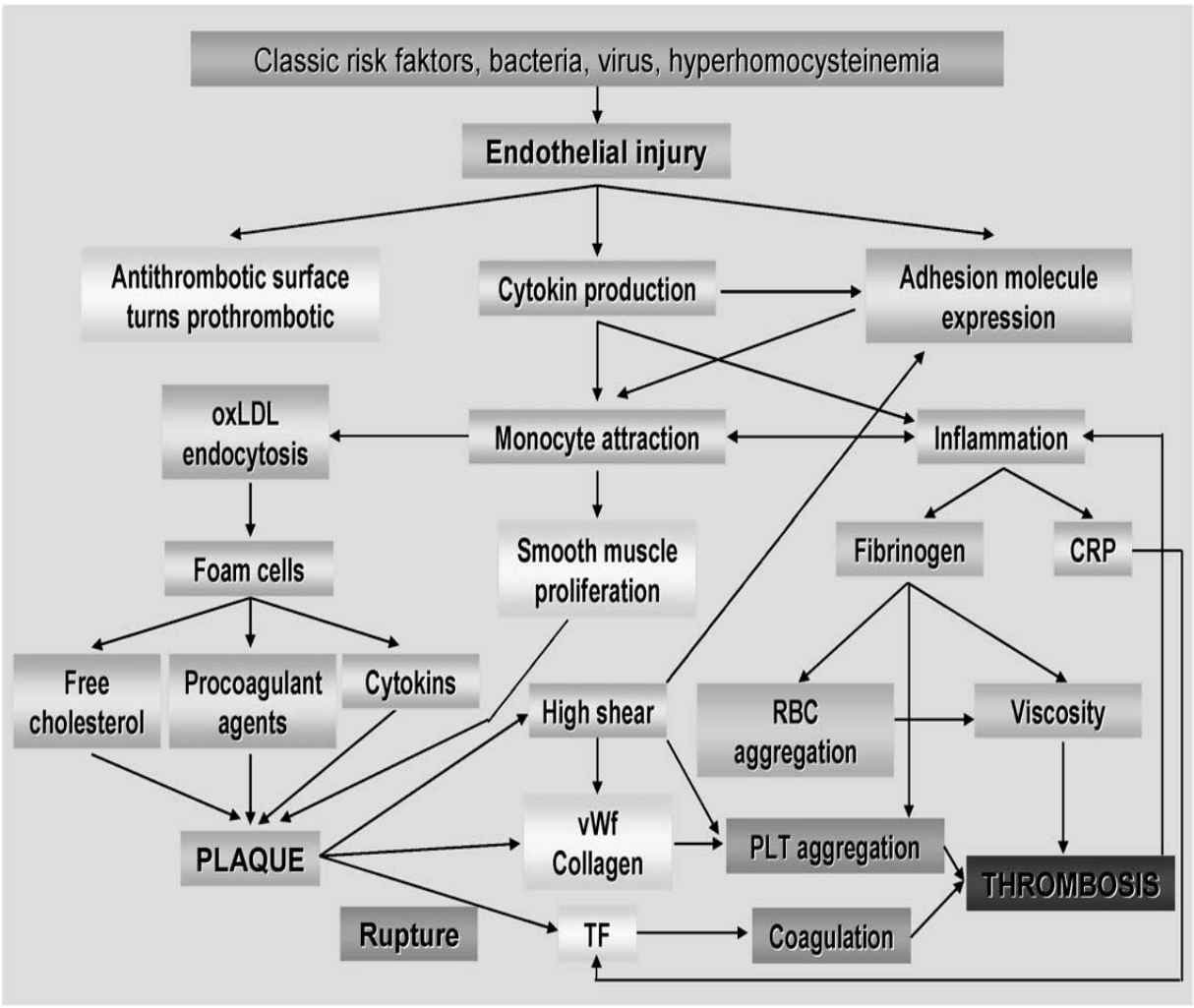

Figure 1: *The role of hemorheology in atherothrombosis.*

Several studies confirmed that hemorheological parameters are independent risk factors of ischemic heart disease (IHD) (7-10). While the role of "classic" risk factors has been noted a long time ago, recent clinical and epidemiological studies have provided compelling evidence that altered hemorheological parameters are also primary risk factors for the development and progression of cardio- and cerebrovascular diseases. Identifying elevated whole blood viscosity is of superior clinical importance since it has a predictive value for increased resistance to blood flow in the general circulation and for abnormal hemodynamics

in the microcirculation. Hemorheological parameters play a role in the development of ischemic areas causing insufficient circulation, local stasis, capillary clogging, thrombus formation, slow O_2 transport and hypoxia. It promotes atherosclerotic plaque formation and shear stress damage at the endothelial wall leading to occlusive thrombus formation (7-10) (see Figure 1). „Classic" risk factors can also influence hemorheological parameters. A positive correlation between body mass index (BMI) and blood viscosity and its determinants has been demonstrated in several studies (11). Arterial hypertension (AHT) is in association with increased blood viscosity (12). Smoking increases in a reversible way plasma and whole blood viscosity, partly by increasing hematocrit and fibrinogen (9).

Since 1993, results of a meta-analysis of six prospective epidemiological studies, including 14 988 persons for a total of 92 147 person-years investigated, showed that plasma fibrinogen is a powerful independent predictor of myocardial infarction and stroke (10). Afterwards, several prospective trials confirmed that increased whole blood viscosity, primarily dependent upon hematocrit value and fibrinogen concentration, is a major risk factor for ischemic heart disease and stroke (11-13). The relationships of these rheological variables to cardiovascular events are at least as strong as those of conventional risk factors (smoking habit, diastolic blood pressure, and low-density lipoprotein cholesterol) (13).

At the same time, many investigations found that plasma fibrinogen concentration rises with advancing age (14-16). The Italian multicenter Study on Centenarians confirmed this result in a representative population group of very elderly.

A few studies explored the relationship between rising age and whole blood viscosity. Nevertheless, some reports, based on the results of investigations carried out on very small population samples, tend to state that elderly healthy subjects display increased whole blood viscosity, but the results are still controversial (17,18).

1.2 Platelet aggregation and inhibition in atherothrombosis

Platelets are vital components of normal hemostasis and key participants in pathologic thrombosis by virtue of their capacity to adhere to injured blood vessels and to accumulate at sites of injury (19). Although platelet adhesion and activation should be viewed as a 'physiological' response to the sudden fissuring or rupture of an atherosclerotic plaque, eventually contributing to its repair, uncontrolled progression of such a process through a series of self-sustaining amplification loops may lead to intraluminal thrombus formation,

vascular occlusion and transient ischemia or infarction (see Figure 2). Currently available antiplatelet drugs interfere with some steps in the activation process, including adhesion, release, and/or aggregation, and have a measurable impact on the risk of arterial thrombosis that cannot be dissociated from an increased risk of bleeding (20).

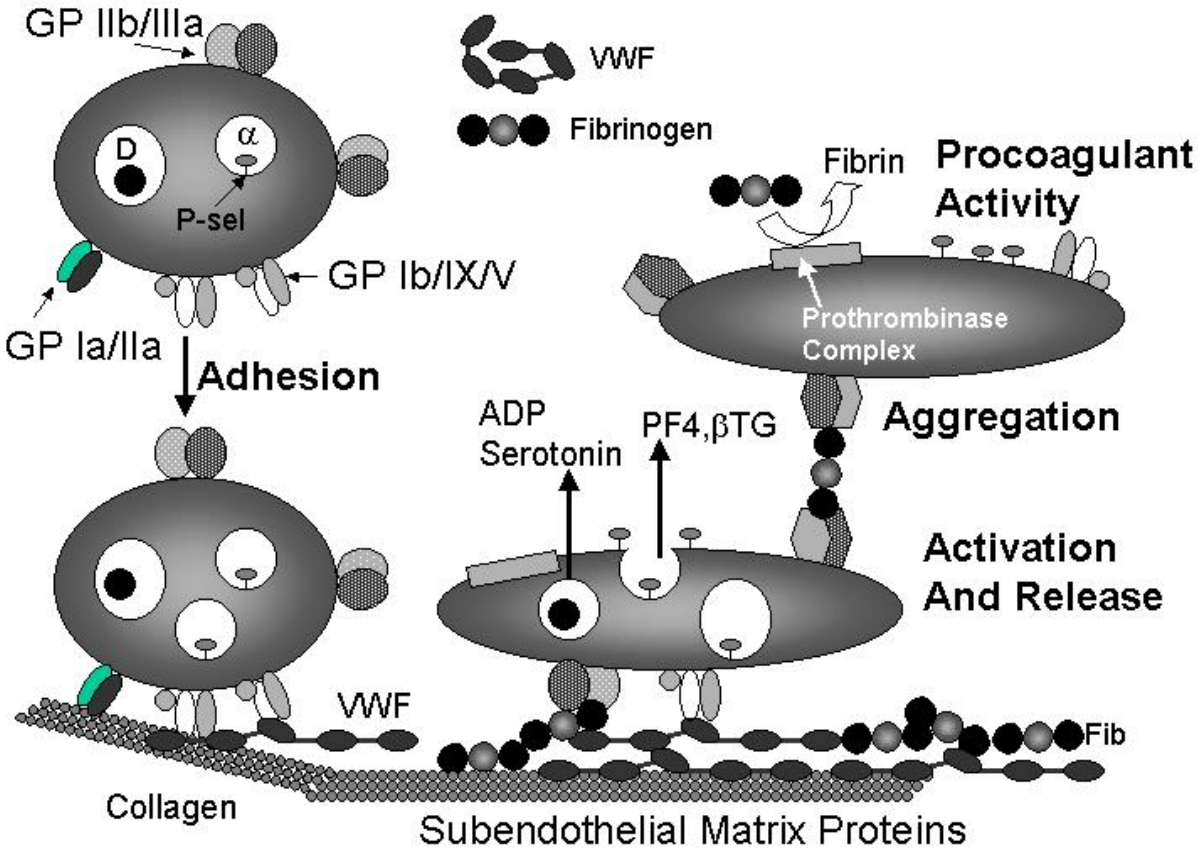

Figure 2: *Platelet adhesion, activation and aggregation.*

In discussing antiplatelet strategies, it is important to recognise that approximately 1000 platelets are produced each day under physiological circumstances, a level of production that can increase up to tenfold at times of increased need (21). Platelets form by fragmentation of megakaryocyte cytoplasm and have a maximum circulating life span of about 10 days in man (21). Thus, platelets are anucleate blood cells that provide a circulating source of chemokines, cytokines and growth factors that are preformed and packaged in storage granules. Moreover, activated platelets can synthesize prostanoids [primarily, thromboxane (TX)A2] from arachidonic acid released from membrane phospholipids, through rapid

coordinated activation of phospholipase(s), cyclo-oxygenase (COX)-1 and TX-synthase3. Newly formed platelets also express the inducible isoforms of COX (COX-2) and prostaglandin E (PGE)-synthase, and this phenomenon is markedly amplified in association with accelerated platelet regeneration (22). Although activated platelets are not thought to synthesize proteins de novo, they can translate constitutive mRNAs into proteins, including interleukin-1 over several hours (23). Thus, platelets may have previously unrecognized roles in inflammation and vascular injury, and antiplatelet strategies may be expected to impact on platelet-derived protein signals for inflammatory and/or proliferative responses (23,24). Negative modulation of platelet adhesion and aggregation is exerted by a variety of mechanisms, including endothelium-derived prostacyclin (PGI2), nitric oxide, CD39/ecto-ADPase and platelet endothelial cell adhesion molecule-1 (PECAM-1) (24-26). Some drugs may interfere with these regulatory pathways, as exemplified by the dose-dependent inhibition of PGI2 production by aspirin and other COX-inhibitors (19). The apparent redundancy of mechanisms of endothelial thromboresistance is likely to limit the clinical consequences of PGI2 inhibition by COX-inhibitors.

Aspirin induces a long-lasting functional defect in platelets, which can be detectable clinically as a prolonged bleeding time. This appears to be primarily, if not exclusively, due to permanent inactivation by aspirin of a key enzyme in platelet arachidonate metabolism (see Figure 3). This enzyme, prostaglandin (PG) H-synthase, is responsible for the formation of PGH2, the precursor of TXA2. In human platelets, TXA2 provides a mechanism for amplifying the activation signal through its being synthesized and released in response to various platelet agonists (e.g., collagen, adenosine diphosphate [ADP], plateletactivating factor, thrombin) and, in turn, inducing irreversible aggregation (27). Aspirin selectively acetylates the hydroxyl group of a single serine residue at position 529 (Ser529) within the polypeptide chain of platelet PGH-synthase. This enzyme exhibits two distinct catalytic activities: a bis-oxygenase (cyclo-oxygenase [COX]) involved in formation of PGG2, and a hydroperoxidase allowing a net two-electron reduction in the 15-hydroperoxyl group of PGG2, thus yielding PGH2. Through O-acetylation of Ser529 by aspirin, the cyclo-oxygenase activity is lost permanently, whereas the hydroperoxidase activity is not affected. An inducible form of PGH-synthase has been identified and termed PGH-synthase 2 or COX-2 (28). Aspirin inhibits the cyclo-oxygenase activity of PGH-synthase 2, but at higher concentrations than those required to inhibit PGHsynthase 1 or COX-1 (i.e. the constitutive enzyme) (29).

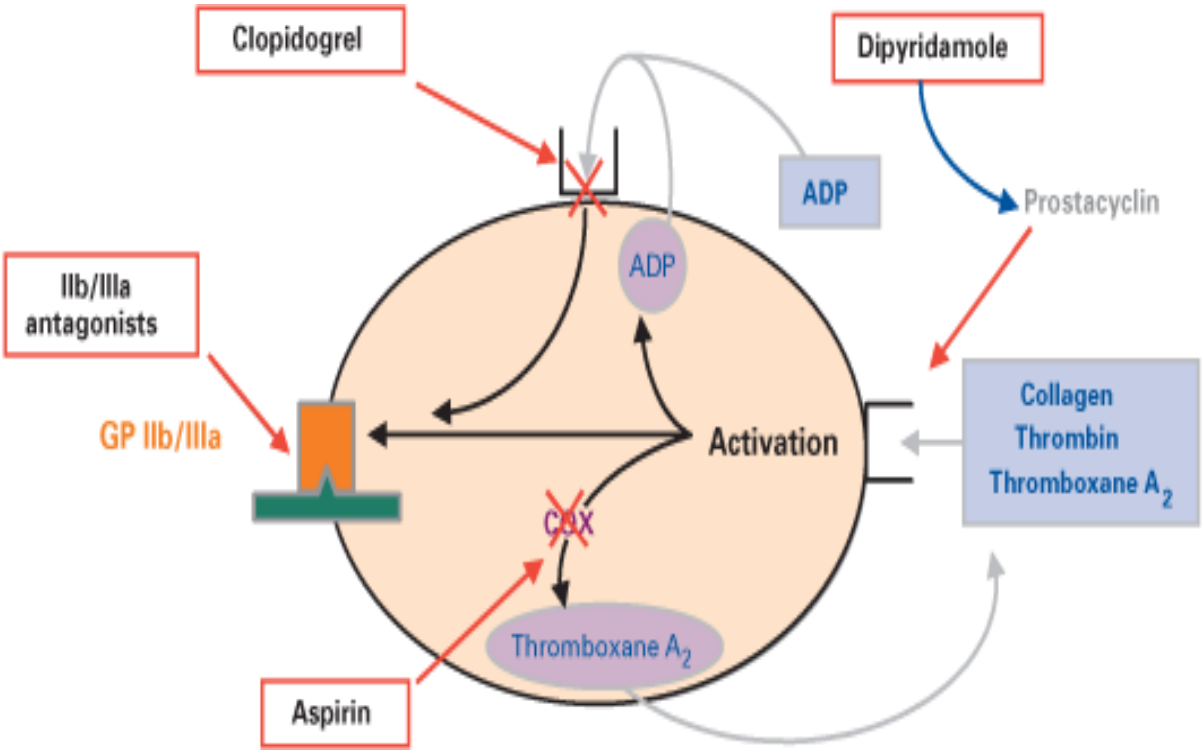

Figure 3: *The antiaggregatory mechanism of widely used antiplatelet agents.*

This may account, at least in part, for the different dose requirements of analgesic and anti-inflammatory versus antiplatelet effects of the drug. A very large database of randomized clinical trials now offers the most compelling evidence that prevention of myocardial infarction and ischemic stroke by aspirin is largely due to permanent inactivation of platelet COX-1 (19). These studies, which tested the efficacy and safety of the drug when given at daily doses ranging from as low as 30 mg to as high as 1500 mg, have established two important facts. First, the anti-thrombotic effect of aspirin is saturable at doses in the range of 75 to 100 mg, as would be expected from human studies of platelet COX-1 inactivation (27). Second, despite a half-life of approximately 20 min in the human circulation, the anti-thrombotic effect of aspirin is observed with dosing intervals of 24 to 48 hours, reflecting the permanent nature of platelet COX-1 inactivation and the duration of TXA2 suppression following oral dosing in man (27). Other mechanisms of action that have been suggested to contribute to the anti-thrombotic effect of aspirin, such as an anti-inflammatory effect of the drug, are simply incompatible with these unique properties. Although the search for the lowest effective dose of aspirin for platelet inhibition was largely driven by the explicit

concern of concomitant inhibition of vascular PGI2 production, it is still uncertain whether dosedependent suppression of the latter attenuates the anti-thrombotic effect of aspirin in clinical syndromes of vascular occlusion (27). The biochemical selectivity of low dose aspirin arises from both pharmacokinetic determinants, such as the acetylation of platelet COX-1 that occurs in portal blood (prior to first-pass metabolism), and pharmacodynamic determinants, such as the limited sensitivity of endothelial COX-2 to the drug. Aspirin is an effective anti-thrombotic agent in a wide range of daily doses. Whether dose-dependent inhibition by aspirin of a mediator of thromboresistance, such as PGI2, may be responsible for a somewhat attenuated efficacy at high daily doses remains to be demonstrated convincingly (19,27,28).

Aspirin's unique feature in inhibiting platelet COX-1 (i.e. its ability to inactivate the enzyme permanently through a short-lived active moiety) is ideally suited to its role as an antiplatelet drug, because they severely limit the extent and duration of extraplatelet effects of the drug, including the inhibition of PGI2. Moreover, the cumulative nature of platelet COX-1 acetylation by repeated low doses of aspirin16 explains the clinical efficacy of doses as low as 30 to 50 mg daily, the predictable high-grade inhibition of platelet TXA2 biosynthesis, and the persistence of the drug's effect. These features, in turn, may limit the consequences of less-than-ideal compliance in a real world setting. Permanent inactivation of platelet COX-1 by aspirin may lead to the prevention of thrombosis as well as to excess bleeding. At least two distinct COX-1-dependent mechanisms contribute to the increased risk of upper GI bleeding associated with aspirin exposure: inhibition of TXA2-mediated platelet function and impairment of PGE2-mediated cytoprotection in the gastrointestinal (GI) mucosa (19,27). Whereas the former effect is dose independent, at least for daily doses > 30 mg, the latter effect is clearly dose-dependent. Inhibition of platelet function is largely responsible for the 2-fold increase in the risk of upper GI bleeding associated with daily doses of aspirin in the range of 75 to 100 mg, in as much as a similar relative risk is associated with other antiplatelet agents that do not act on COX and therefore do not affect PGE2-mediated cytoprotection (29,30). Inhibition of COX-1-dependent cytoprotection amplifies risk of bleeding/ perforation by causing new mucosal lesions or aggravating existing ones, and is associated with a relative risk of four to six at the higher, analgesic or antiinflammatory doses of aspirin. Assessing the net effect of aspirin requires an estimation of the absolute risk of the individual patient for thrombotic or hemorrhagic complications. In individuals at very low risk for vascular occlusion (i.e. less than 1% per year), a very small absolute benefit may be offset by exposure of very large numbers of healthy subjects to undue serious bleeding complications. As the risk

of experiencing a major vascular event increases, so does the absolute benefit of antiplatelet prophylaxis with aspirin and, above a certain threshold, benefit clearly outweighs risk of bleeding (19,27).

Clopidogrel is structurally related the thienopyridines with platelet inhibitory properties. This drug selectively inhibits ADP-induced platelet aggregation, with no direct effects on the metabolism of arachidonic acid (19). Clopidogrel can also inhibit platelet aggregation induced by collagen and thrombin, but this inhibitory effect is abolished by increasing the agonist concentration and, therefore, likely reflect blockade of ADP-mediated amplification of the response to other agonists. Clopidogrel do not affect ADP-induced platelet aggregation when added in vitro up to 500 μM, thus suggesting that in vivo hepatic transformation to an active metabolite, or metabolites, is necessary for its antiplatelet effects. A short-lived, active metabolite of clopidogrel has been characterized (31). Recent evidence suggests that clopidogrel and, probably, ticlopidine induce irreversible alterations of the platelet ADP receptor P2Y12 mediating inhibition of stimulated adenylyl cyclase activity by ADP (32) (see Figure 3). Inhibition of platelet function by clopidogrel is associated with a selective reduction in ADP-binding sites, with no consistent change in the binding affinity. Permanent modification of an ADP receptor by thienopyridines is consistent with time-dependent, cumulative inhibition of ADP-induced platelet aggregation on repeated daily dosing and with slow recovery of platelet function on drug withdrawal (19).

After single oral doses of clopidogrel, ADP-induced platelet aggregation was inhibited in a dose-dependent fashion in healthy volunteers, with an apparent ceiling effect (i.e. 40% inhibition) at 400 mg. Inhibited platelet aggregation was detectable 2 hours after oral dosing of 400 mg, and it remained relatively stable up to 48 hours. With repeated daily dosing of 50 to 100 mg in healthy volunteers, ADP-induced platelet aggregation was inhibited from the second day of treatment (25–30% inhibition) and reached a steady state (50–60% inhibition) after 4 to 7 days. Such maximal inhibition is comparable to that achieved with ticlopidine (500 mg daily). Ticlopidine, however, has shown a slower onset of antiplatelet effect compared with clopidogrel (33).

The best available interpretation of these findings is that the active metabolite of clopidogrel has a pharmacodynamic pattern quite similar to that of aspirin in causing cumulative platelet inhibition on repeated daily low-dose administration (19,33). As with aspirin, platelet function returned to normal 7 days after the last dose. Both the cumulative nature of the inhibitory effects and the slow recovery rate of platelet function are consistent

with the active moieties of aspirin (i.e. acetylsalicylic acid) and clopidogrel (i.e. active metabolite) causing a permanent defect in a platelet protein that cannot be repaired during the 24-h dosing interval and can be replaced only through platelet turnover (33). This also justifies the oncedaily regimen of both drugs despite their short half-life in the circulation. Bleeding times measured in the same multiple-dose study of clopidogrel described earlier showed a comparable prolongation (by 1.5–2.0-fold over controls) at 50 to 100 mg daily (19,33).

Clopidogrel has undergone an unusual clinical development, with limited phase II studies and a single, large phase III trial (i.e. CAPRIE) to test its efficacy and safety at 75 mg daily compared with aspirin at 325 mg daily. Clopidogrel was slightly more effective than aspirin, and there was some suggestion from a marginally significant heterogeneity test that clopidogrel may be particularly effective at preventing vascular events in patients with symptomatic peripheral arterial disease. This interesting and, perhaps, unexpected finding suggests that the pathophysiologic importance of TXA2 and ADP varies in different clinical settings. In the CAPRIE trial, the frequency of severe rash was higher with clopidogrel than with aspirin (absolute excess approximately 1–2 per 1000), as was the frequency of diarrhoea, thus reproducing the characteristic side effects of ticlopidine. No excess neutropenia, however, was associated with clopidogrel, the frequency of this serious complication was extremely low (0.05 %) in this trial (34). The CURE trial has demonstrated the efficacy and safety of adding clopidogrel (a loading dose of 300 mg, followed by 75 mg daily) to aspirin in the long-term management of patients with acute coronary syndromes without ST-segment elevation (35). Moreover, the combination of aspirin and clopidogrel has become standard treatment for 1 month after coronary stent implantation. The recently reported CREDO trial has demonstrated that following percutaneous coronary interventions, long-term (1-year) clopidogrel therapy significantly reduces the risk of adverse ischemic events (19,33-35).

Aspirin and clopidogrel have emerged as critical therapies in the treatment of cardiovascular diseases. Despite their efficacy, patients on these medications continue to suffer complications. Millions of patients are currently on low-dose antiplatelet therapy, but it is unknown how many of these patients are under-treated or on the wrong medication. The term “aspirin resistance” or “clopidogrel resistance” has been used in a clinical and laboratory context. ‘Clinical aspirin resistance’ or ‘clinical clopidogrel resistance’ have been used to refer to the inability of these drugs to protect individuals from cardiovascular thrombotic events such as acute MI. Furthermore, the diagnosis of clinical resistance can only be made in

retrospect because an ischemic event must occur before a diagnosis of clinical resistance can be considered. ‘Laboratory aspirin resistance’ or ‘laboratory clopidogrel resistance’ refer to the lack of an anticipated effect of these antiplatelet drugs on a laboratory measure of its antiplatelet effect that is associated with a higher rate of future cardiovascular events (37-39).

As the mechanisms of aspirin and clopidogrel resistance are becoming clearer, defining these clinical entities remains a challenge. The lack of a standard definition of resistance as well as the lack of a standard diagnostic modality have hampered the field in identifying and treating this clinical entity. Attempts have been made to develop a more meaningful definition with the goal of correlating laboratory tests with clinical outcomes, but there is no current definition which unifies the biochemical and clinical expression of failed treatment. Rates of aspirin resistance range from 5 to 45% of the population depending on the study and the method of determining therapeutic failure. Rather than attempting to characterize patients as simply resistant or sensitive to a medication, however, therapeutic resistance is more likely a continuous variable similar to blood pressure. By shifting the paradigm of diagnosis from a specific value to this notion of a continuous variable, the physiology of treatment failure may be better elucidated and thus better managed. In the meantime, a clear and consistent characterization of antiplatelet resistance is necessary (37-39).

2. AIMS OF THE INVESTIGATIONS

1. The aim of our first study was to examine the relationship of hemorheological parameters to the “classic” risk factor, advancing age.

2. The aim of our second study was to compare some parameters of patients with effective platelet inhibition by aspirin (ASA) (risk profile, previous diseases, medication, and hemorheological parameters) to patients with ineffective ASA treatment.

3. The aim of our third study was to compare some parameters of patients with effective platelet inhibition by clopidogrel (CLP) (risk profile, previous diseases, medication, hemorheological parameters, plasma von Willebrand factor and soluble P-selectin levels) to patients with ineffective clopidogrel treatment.

3. HEMORHEOLOGICAL PARAMETERS AND AGING

Cardio- and cerebrovascular diseases and their complications are the leading cause of morbidity and mortality in the developed world. While the role of "classic" risk factors (e.g. smoking, hypertension, hypercholesterolemia, diabetes, etc.) has been well known for a long time, the importance of hemorheologic parameters in the development of coronary artery disease (CAD) has been accepted only in the last two decades. „Classic" risk factors can also influence hemorheological parameters. A positive correlation between body mass index (BMI) and blood viscosity and its determinants has been demonstrated in several studies. Arterial hypertension is in association with increased blood viscosity. Smoking increases in a reversible way plasma and whole blood viscosity, partly by increasing hematocrit and fibrinogen. The relationships of these rheological variables to cardiovascular events are at least as strong as those of conventional risk factors (smoking habit, diastolic blood pressure, and low-density lipoprotein cholesterol).

The aim of our present study was to determine the association between hemorheological parameters (hematocrit, fibrinogen, red blood cell aggregation, plasma and whole blood viscosity) and increasing age, because a relatively few number of studies examined the association between these parameters and increasing age, and their results are controversial.

3.1 Patients and methods

3.1.1 Patients

Between January 1999 and May 2004 the blood samples of 6236 cardio- and cerebrovascular patients (3774 males, mean age 59.8+/-13.2 years and 2462 females, mean age 60.9+/-12.8 years) were examined in our laboratory. Both sexes were divided into three categories: A. young patients: <45 years. B, middle-aged patients: 45-65 years and C, old patients: >65 years. To evaluate the possible effect of risk profile, 623 patients from the study population with matching parameters were divided into the three age groups as mentioned before. They had the same prevalence of risk factors including body mass index, smoking habits, diabetes mellitus, dyslipidemia, hypertension), previous diseases (pulmonary embolism, deep vein thrombosis, ischemic heart disease (IHD), acute myocardial infarction (AMI), stenosis of the carotid artery, transient ischemic attack (TIA), stroke and peripheral arterial disease and took the same medication beta-blockers, alpha blockers, angiotensine converting enzyme (ACE)

inhibitors, angiotensine II (AT II) receptor antagonists, calcium (CA) channel blockers, nitrates, statins, fibrates, diuretics, H2 receptor blockers, proton pump inhibitors (PPI), acetylsalicylic acid (ASA), clopidogrel (CLP), trimetazidine (TMZ), vinpocetine and pentoxyphylline (see Table 1).

3.1.2. Methods

3.1.2.1 Hemorheological measurements

Blood samples were taken from the cubital vein; routine blood chemistry and hemorheological parameters - hematocrit, plasma fibrinogen level, plasma and whole blood viscosity, red blood cell (RBC) aggregation and deformability - were determined.

Hematocrit:

Venous blood collected into lithium-heparin coated Vacutainer tubes was used to determine hematocrit. Hematocrit was measured by centrifuging hematocrit capillaries (80 µl, containing heparin) at 12000 rpm for five minutes in microhematocrit centrifuge (Hemofuge, Heraeus Instr., Germany). Measurements were performed at room temperature (22 ± 1 ºC).

Plasma fibrinogen:

4.5 ml blood sample was drawn into a Vacutainer tube containing sodium citrate (0.129 M, 1:10 dilution) and plasma fibrinogen concentration was determined by using Clauss's method (40).

Plasma and whole blood viscosity:

Venous blood samples were collected into lithium-heparin coated Vacutainer tubes for viscosity measurements. Plasma was prepared by centrifuging samples at 1500 g for ten minutes. Plasma and whole blood viscosities were determined in Hevimet 40 capillary viscosimeter (Hemorex Ltd., Hungary). 1.0-1.0 ml of plasma or whole blood was injected into the capillary tube of the device. In this viscosimeter the flow of the fluid is detected optoelectronically along the capillary tube and a flow curve is drawn. Shear rate and shear stress are calculated from this curve, viscosity values are determined as a function of these parameters according to Casson's principle (41). For the presentation of our results, apparent whole blood viscosity values calculated at 90 s^{-1} shear rate are given. Measurements were carried out at 37.0 ± 0.5 ºC within 2 hours after venepuncture.

	young patients n = 119	middle-aged patients n = 226	old patients n = 278
Mean age (mean ± SEM)	32.4 ± 0.15	54.1 ± 0.1	72.4 ± 0.12
BMI (kg/m^2) (mean ± SEM)	26.95 ± 0.08	27.11 ± 0.06	27.32 ± 0.05
Smoking (%)	34	31	32
Diabetes mellitus (%)	46	49	45
Hyperlipidemia (%)	44	41	42
Hypertension (%)	60	62	65
Pulmonary embolism (%)	5	6	4
Deep vein thrombosis (%)	9	11	8
IHD (%)	32	31	34
AMI (%)	15	17	19
Stenosis of the carotid artery (%)	5	6	8
TIA (%)	2	3	2
Stroke (%)	12	14	13
Peripheral arterial disease (%)	13	11	14
β-blockers (%)	42	40	41
α-blockers (%)	12	14	10
ACE-inhibitors (%)	45	48	49
AT-II-receptor blockers (%)	4	3	3
Ca-channel blockers (%)	30	28	31
Nitrates (%)	12	13	13
Statins (%)	26	28	30
Fibrates (%)	2	3	3
Diuretics (%)	12	14	15
H2-receptor blockers (%)	18	19	18
PPI (%)	22	23	25
ASA (%)	55	56	56
CLP (%)	12	13	11
TMZ (%)	18	19	19
Vinpocetine (%)	19	17	16
Pentoxyphilline (%)	8	11	12

Table 1: *Baseline characteristics in the selected population*

RBC aggregation:

RBC aggregation measurements were carried out from venous blood samples collected into lithium-heparin coated Vacutainer tubes. RBC aggregation was measured in Myrenne aggregometer (MA-1 Aggregometer, Myrenne GmbH, Germany) applying the light transmission method. In this study the aggregometer was used in low shear (M1) mode. In this

mode blood sample (30 μl) is first sheared at 600 s^{-1} to disperse all pre-existing aggregates, then shear rate decreases rapidly to low shear (3 s^{-1}). The extent of aggregation is characterized by the aggregation index (AI), calculated from the surface area below the light intensity curve in a 10 s measurement period (42,43). Measurements were performed at room temperature (22 ± 1 ºC) within 2 hours after venepuncture.

3.1.2.2. Statistical analysis

Data were evaluated as means ± S.E.M. (standard error of mean) by Student's t test and chi square test.

3.2 Results

3.2.1 Whole population

3.2.1.1 Hematocrit

Hematocrit level slightly, but significantly increased with advancing age ($p < 0.01$) in the whole population as well as in males and females. Analyzing the data of the different age subgroups we found that hematocrit increased with age in the young and middle-aged males ($p < 0.05$), while it decreased significantly in the old patients ($p < 0.01$). On the other hand, in females a significant positive correlation could only be found in young patients ($p < 0.01$). Young patients had significantly lower hematocrit levels ($p < 0.01$) compared to the other two age groups. There was no difference between middle-aged and old patients in either sexes.

3.2.1.2 Plasma fibrinogen

Plasma fibrinogen significantly increased with age ($p < 0.01$) in the studied population and also in both genders ($p < 0.05$). In the different age subgroups positive correlation could be found in old males and middle-aged females ($p < 0.01$). Young patients had significantly lower plasma fibrinogen levels ($p < 0.01$) compared to the other two age groups.

3.2.1.3. Red blood cell aggregation

There was a significant correlation between red blood cell aggregation and advancing age in both males and females ($p < 0.05$). Analyzing the subgroups, we found a significant positive correlation in young males and significantly decreasing values in the case of old males ($p < 0.05$). Young patients had significantly lower red blood cell aggregation level compared to the other groups ($p < 0.001$).

3.2.1.4. Plasma viscosity

Plasma viscosity significantly increased with age when both sexes were considered together ($p < 0.01$). This finding remained significant in males, but not in females ($p < 0.01$).

	whole population n = 6236	males n = 3774	females n = 2462	young males n = 309	middle-aged males n = 1687	old males n = 1778	young females n = 212	middle-aged females n = 1098	old females n = 1152
mean age (mean ± SEM)	60.3 ± 0.03	59.8 ± 0.03	60.9 ± 0.05	36.1 ± 0.1	56.8 ± 0.07	70.8 ± 0.06	35.1 ± 0.08	58.5 ± 0.03	70.9 ± 0.03
hematocrit	r=0.049**	r=0.069**	r=0.066**	r=0.147**	r=0.074*	r=-0.11**	r=0.255**	r=-0.015	r=-0.064
fibrinogen	r=0.108**	r=0.105**	r=0.077*	r=0.068	r=0.053	r=0.99**	r=-0.011	r=0.105**	r=0.036
red blood cell aggregation index	r=0.098**	r=0.102**	r=0.09*	r=0.398**	r=-0.05	r=-0.085*	r=0.05	r=0.002	r=-0.093
plasma viscosity	r=0.148**	r=0.18**	r=0.085	r=0.159**	r=0.032	r=0.031	r=-0.011	r=0.039	r=0.034
whole blood viscosity	r=0.083**	r=0.124**	r=0.047	r=0.117	r=0.042	r=-0.101**	r=0.05	r=0.001	r=-0.045

Table 2: *Correlation coefficient (2-tailed) and its significancy in the whole population (*correlation is significant at the 0.05 level (2-tailed), ** is significant at the 0.01 level)*

	males	females	young males	middle-aged males	old males	young females	middle-aged females	old females
hematocrit (%)	44.79 ± 0.4	41.88 ± 0.3	43.6 ± 0.3 *	45.1 ± 0.2	44.9 ± 0.3	40.22 ± 0.4 *	42.3 ± 0.4	41.8 ± 0.3
fibrinogen (g/l)	3.392 ± 0.06	3.429 ± 0.07	3.28 ± 0.08	3.38 ± 0.08	3.44 ± 0.07	3.07 ± 0.09	3.43 ±0.11	3.48 ± 0.1
red blood cell aggregation index	25.21 ± 0.32	25.41 ± 0.33	22.69 ± 0.3 **	25.89 ± 0.34	25.36 ± 0.30	23.06 ± 0.4 **	25.68 ± 0.36	25.60 ± 0.27
plasma viscosity (mPas)	1.28 ± 0.01	1.30 ± 0.01	1.22 ± 0.01 '	1.29 ± 0.01	1.29 ± 0.01	1.27 ± 0.01 '	1.31 ± 0.01	1.30 ±0.01
whole blood viscosity (mPas)	4.79 ± 0.04	4.42 ± 0.06	4.46 ± 0.04 *	4.88 ± 0.04	4.83 ± 0.05	4.27 ± 0.04 *	4.47 ± 0.06	4.41 ± 0.07

Table 3: *Mean values ± SEM in the whole population (' p < 0.05, * p<0.01, ** p< 0.001)*

Examining the age groups we could find a significant correlation only in young men ($p < 0.01$). Lower plasma viscosity values could be found in young patients compared to the other groups ($p < 0.05$).

3.2.1.4. Whole blood viscosity

Whole blood viscosity significantly increased with age in the whole population ($p < 0.01$), but analyzing the sex groups we could find a significant negative correlation only in old males ($p < 0.01$). Young patients had lower whole blood viscosity values than middle-aged and old patients ($p< 0.01$) (see Tables 3 and 4).

3.2.2 Selected population

All the measured parameters did not correlate with increasing age neither in the selected population nor in the subgroups. No significant difference could be found in the mean values of the different subgroups (see Tables 4 and 5).

	Whole population n = 623	males n = 309	females n = 226	young males n = 75	middle-aged males n = 146	old males n = 176	young females n = 44	middle-aged females n = 80	old females n = 102
hematocrit	r = 0.063	r = 0.024	r = 0.047	r = 0.098	r = 0.042	r = - 0.051	r = 0.078	r = 0.01	r = - 0.015
fibrinogen	r = 0.14	r = 0.124	r = 0.147	r = 0.155	r = 0.07	r = - 0.02	r = 0.138	r = 0.098	r = 0.017
red blood cell aggregation index	r = 0.089	r = 0.104	r = 0.067	r = 0.11	r = 0.047	r = - 0.072	r = 0.10	r = 0.038	r = - 0.03
plasma viscosity	r = 0.067	r = 0.074	r = 0.047	r = 0.052	r = 0.037	r = 0.022	r = 0.079	r = 0.018	r = 0.035
whole blood viscosity	r = 0.056	r = 0.064	r = 0.029	r = 0.060	r = 0.017	r = - 0.062	r = 0.092	r = 0.035	r = - 0.028

Table 4: *Correlation coefficient and its significancy in the homogenous population (*correlation is significant at the 0.05 level (2-tailed), ** is significant at the 0.01 level)*

	males	females	young males	middle-aged males	old males	young females	middle-aged females	old females
hematocrit (%)	44.2 ± 0.5	41.6 ± 0.3	43.8 ± 0.3	44.3 ± 0.2	44.5 ± 0.3	41.01 ± 0.4	41.8 ± 0.4	41.9 ± 0.3
fibrinogen (g/l)	3.35 ± 0.04	3.36 ± 0.07	3.33 ± 0.09	3.37 ± 0.07	3.44 ± 0.06	3.29 ± 0.1	3.32 ±0.1	3.38 ± 0.1
red blood cell aggregation index	24.5 ± 0.3	24.8 ± 0.31	24.1 ± 0.25	25.3 ± 0.35	25.3 ± 0.30	24.5 ± 0.31	24.9± 0.3	25.1 ± 0.2
plasma viscosity (mPas)	1.27 ± 0.01	1.28 ± 0.01	1.25 ± 0.01	1.29 ± 0.01	1.29 ± 0.01	1.28 ± 0.01	1.31 ± 0.01	1.30 ±0.01
whole blood viscosity (mPas)	4.68 ± 0.04	4.46 ± 0.06	4.58 ± 0.04	4.68 ± 0.04	4.73 ± 0.05	4.47 ± 0.04	4.47 ± 0.06	4.46 ± 0.07

Table 5: *Mean values ± SEM in the homogenous population*

*(' p < 0.05, * p<0.01, ** p< 0.001)*

DISCUSSION

Since 1993, results of a meta-analysis of six prospective epidemiological studies, including 14 988 persons for a total of 92 147 person-years investigated, showed that plasma fibrinogen is a powerful independent predictor of myocardial infarction and stroke (10). Afterwards, several prospective trials confirmed that increased whole blood viscosity, primarily dependent upon hematocrit value and fibrinogen concentration, is a major risk factor for ischemic heart disease and stroke (11-13). The relationships of these rheological variables to cardiovascular events are at least as strong as those of conventional risk factors (smoking habit, diastolic blood pressure, and low-density lipoprotein cholesterol) (13).

On the other hand, the role of hemoheological parameters still remains unclear. A number of clinical studies have demonstrated significant positive correlation between the severity of arterial hypertension (AHT) and whole blood viscosity. Red blood cell aggregation has also been associated with AHT especially in the severe form of the disease. The main possible cause of increased red blood cell aggregation is fibrinogen which can be found in a significantly higher concentration in patients with AHT than in healthy controls. On the other hand, blood pressure reduction with angiotensin-converting-enzyme inhibitors, calcium-channel-blocking agents, beta or alpha-receptor blocking drugs leads to a significant improvement of blood rheology. It can be presumed that abnormal hemorheology and AHT are not directly linked but they share the same inductive genetic and/or environmental factors like obesity, chronic mental stress, physical inactivity and cigarette smoking. Regarding this hypothesis, the appropriate question is not whether hemorheological factors are causes or results of AHT but what their common origins are. Further studies are needed to clarify this hypothetical link between hemorheology and AHT (44).

Despite the statistically significant association between hematocrit and coronary heart disease in the general population, the risk ratio is only slightly elevated above 1·0 and its relevance remains uncertain. Hematocrit levels are correlated with a number of standard vascular risk factors and adjustment for the measured values of these factors in some studies reduces the strength of the associations between hematocrit and coronary heart disease. Hence, adjustment for the longterm usual values of those factors (and other possible confounders) should reduce the risk ratio still further towards 1·0. It is also possible that the available evidence on hematocrit and coronary heart disease has been exaggerated somewhat by publication bias. Assays for hematocrit are widely available, so other relevant studies of

hematocrit and incident coronary heart disease may well exist (e.g., in trials of vascular disease prevention) that have not yet been reported. Indeed, separate results for coronary heart disease were not reported in a few long-term prospective studies of hematocrit and all-cause mortality, but any bias owing to the absence of these published studies is not likely to be substantial since they include less than 5% of the deaths in the available studies (45).

A recent meta-analysis used data from the Edinburgh Artery Study, a population cohort study started in 1987 that comprised 1592 men and women aged 55 to 74 years. Subjects were followed for a mean of 17 years, and 416 of them suffered at least 1 cardiovascular event. In analyses adjusted for cardiovascular risk factors and history of cardiovascular disease (CVD): C-reactive protein (CRP), interleukin-6, fibrinogen, fibrin D-dimer, tissue plasminogen activator (t-PA), leukocyte elastase, and lipoprotein(a) (all $p<0.01$), as well as von Willebrand factor and plasma viscosity (both $p<0.05$), had significant hazard ratios for incident CVD. Further adjustment for a measure of subclinical atherosclerosis (ankle brachial index) had little impact on these associations. The hazard ratios (95% CI) for incident CVD between top and bottom tertiles in the latter analysis were 1.78 (1.30 to 2.45) for C-reactive protein, 1.85 (1.33 to 2.58) for interleukin-6, and 1.76 (1.35 to 2.31) for fibrinogen (46).

We examined the possible connection between increasing age and hemorheological parameters in 6234 cardio- and cerebrovascular patients first in the whole study population and later in the different subgroups. We found a very weak but statistically significant positive correlation between increasing age and hemorheological parameters in the whole population, but not in all subgroups. In the case of old males whole blood viscosity and its main determinants were negatively correlated with advancing age. These results suggest that these positive correlations are probably just statistically, but not clinically significant. In the second part of our study, in the selected population we did not find any significant correlation, not even in the subgroups. Our results suggest that these parameters are mostly independent of aging, increased values are not associated with older age but the more frequently occurring diseases.

„Classic" risk factors can also influence hemorheological parameters. A positive correlation between body mass index (BMI) and blood viscosity and its determinants has been demonstrated in several studies (11). Arterial hypertension is in association with increased blood viscosity (12). Smoking increases in a reversible way plasma and whole blood viscosity, partly by increasing hematocrit and fibrinogen (9). Many drugs have potential

effects on hemorheological parameters (47,48). Relatively few study examined the associations between hemorheological parameters and increasing age. They reported a not very pronounced increase and these reports depended on the populations studied and were controversial (49-53). To evaluate the effect of risk profile, previous diseases and medication 623 patients were selected from the examined group with the same parameters.

In the homogenous population we did not find any significant correlation neither in the whole population nor in the subgroups. It is very similar to the findings of Coppola et al. (18) but they found age-related dicrease of hematocrit level in the case of old men, but they examined apparently healthy people who were free from clinically detectable illnesses and their biochemical routine parameters were normal. Our study and a recent study did confirm their findings except for the age-related decrease in hematocrit levels. A recent population based study led to the same result (54).

In our knowledge our study is the first which examined the correlation between hemorheological parameters to advancing age in homogenous vascular diseased patient population. The summary of our study is that these parameters are independent of aging, increased values are not associated with older age but the more frequently occurring diseases.

Finally our study has some limitations. Our database did not contain the laboratory results of our patients. Plasma fibrinogen is also a low grade inflammation marker and is strongly associated with white blood cell count and CRP. Plasma lipids have great influence on plasma viscosity. Further examinations are needed to study their influence on rheological and inflammatory parameters to advancing age in a multivariate analysis.

4. THE POTENTIAL BACKGROUND(S) OF ASPIRIN RESISTANCE

There is no debate that long term aspirin use attenuates the risks of myocardial infarction, stroke, and vascular related deaths in patients with cardiovascular disease, but a significant number of patients prescribed aspirin as antithrombotic therapy have major adverse vascular related events each year. Consequently other antiplatelet agents in addition to aspirin have been prescribed for certain patients (37,39).

The major controversy about aspirin therapy is why particular patients do not benefit from such therapy and how they might be identified. It has been suggested that some patients require a higher dose of aspirin than is normally recommended to achieve the expected antiplatelet effect - for example, inhibition of platelet function or inhibition of platelet thromboxane A_2 synthesis. It is unclear whether these patients simply receive too low aspirin dose, are not compliant, have differing abilities to absorb aspirin, or have an underlying genetic disposition that renders aspirin ineffective. Such patients have been labelled aspirin "resistant" - that is, their platelets are not affected in the same way or are affected differently from the platelets of those who seem to benefit from aspirin therapy (aspirin "sensitive" patients with no subsequent adverse cardiovascular event). Little consistency exists about which measure should be used to identify patients who seem to be resistant to aspirin. Also, few studies have assessed the effect of aspirin resistance on clinically important outcomes (37,39).

In the following experiments we examined the potential factors of aspirin resistance (ASA).

4.1 Patients and methods

4.1.1 Patients

Between March 2004 and April 2005, 99 consecutive chronic cardio- and cerebrovascular patients took part in our study. These patients were commenced on standard antiplatelet therapy with aspirin 100–325 mg/day. Patients were excluded from the study if they were taking any other antiplatelet or anticoagulant medications, had a history of other nonvascular diseases (e.g. hematological, endocrinological disorders or had recently experienced an acute vascular event (AMI, stroke or peripherial embolisation 3 months prior to the study).

4.1.2 Methods

4.1.2.1 Platelet aggregation

Epinephrine-, ADP- and collagen-stimulated aggregation of platelets was analyzed. 450 µl platelet rich plasma (PRP) was measured against 450 µl platelet poor plasma (PPP) to determine spontaneous platelet aggregation. 50 µl of ADP (5 and 10 µM), epinephrine (10 µM) or collagen (2 µg/ml) was added to PRP so as to measure stimulated platelet aggregation. Platelet aggregation was measured using a Carat TX4 optical platelet aggregometer (Carat Diagnostics Ltd., Hungary). Platelet aggregation index has a "normal range" (mean ± 2 SD) to each stimulants in 68 untreated healthy persons (30 males, 38 females, mean age 38 ± 6 years). If a platelet aggregation inhibitor drug is effective, it decreases the aggregation index induced by the appropriate agonists (55,56). ADP stimulation is used to estimate the effect of thienopyridines (ticlopidine and clopidogrel). In our study antiplatelet medication was considered to be effective if the aggregation index to the appropriate agonists (collagen 2 µg/ml and epinephrine 10 µM) were lower than the range of untreated persons (both < 60 %) (56).

4.1.2.2. Hemorheological measurements

Blood samples were taken from the cubital vein; routine blood chemistry and hemorheological parameters - hematocrit, plasma fibrinogen level, plasma and whole blood viscosity, RBC aggregation and deformability - were determined. Details can be seen under 3.1.2 section on page 15-17.

4.1.2.3. Risk profile and previous diseases

Risk profile and investigated previous diseases included body mass index (BMI), smoking habits, diabetes, hypertension, dyslipidemia, deep vein thrombosis, pulmonary embolism, ischemic heart disease (IHD), acute myocardial infarction (AMI), carotid stenosis, transient ischemic attack (TIA), stroke, and peripheral arterial disease. Exclusion criteria included previous acute myocardial infarction or stroke < 3 months, or documented IHD or cerebrovascular disease lasting < 1 year previous to our investigation, and any other than vascular disorders. Hypertension was diagnosed in the case of elevated blood pressure values (>140/90 mm Hg measured twice in a resting position) and in subjects with normal blood pressure (<140/90 mm Hg) but on antihypertensive therapy. Dyslipidemia was defined according to the following criteria: serum total cholesterol level >200 mg/dL, serum high density lipoprotein cholesterol level <40 mg/dL, or serum triglyceride level >195 mg/dL. The term "current smoker" was applied if the patient smoked at least 10 cigarettes a day or

consumed an equivalent daily dose of tobacco. Obesity was defined as a body mass index (BMI) >30 kg/m2. Diabetes mellitus was defined as fasting glucose > 130 mg/dL or patients with normal fasting blood glucose level but on antidiabetic medication.

	ineffective inhibition of platelet aggregation (n = 132)	effective inhibition of platelet aggregation (n = 467)	p value
Mean age (yrs)	62 ± 0.7	64 ± 0.7	n.s.
Female/male ratio	0,48	0,52	n.s.
Mean dosage (mg/daily)	123 ± 0,2	127 ± 0,3	n.s.
Hypertension (%)	62	80	$p < 0.05$
Dyslipidaemia (%)	45	49	n.s
Deep vein thrombosis (%)	9	11	n.s
Pulmonary embolism (%)	5	6	n.s
IHD (%)	51	49	n.s
AMI (%)	25	27	n.s
Stenosis of the carotid artery (%)	11	12	n.s
TIA (%)	11	8	n.s
Stroke (%)	27	25	n.s
Peripheral arterial disease (%)	13	11	n.s
β-blockers (%)	55	75	$p < 0.05$
α-blockers (%)	12	14	n.s
ACE-inhibitors (%)	52	70	$p < 0.05$
ATII -receptor blockers (%)	3	4	n.s
Ca-channel blockers (%)	33	28	n.s
Statins (%)	52	38	$p < 0.05$
Fibrates (%)	4	3	n.s
Diuretics (%)	22	24	n.s
Nitrates (%)	25	23	n.s
H2-receptor blockers (%)	22	24	n.s
PPI (%)	24	27	n.s
TMZ (%)	33	29	n.s
Benzodiazepines (%)	18	18	n.s
SSRIs (%)	11	13	n.s
Phosphodiesterase inhibitors (%)	18	20	n.s
NSAIDs (%)	15	17	n.s

Table 6: *Baseline characteristics of the study groups*

4.1.2.4. History of medication

History of medication was related to beta-blockers, alfa-blockers, angiotensine converting enzyme (ACE) inhibitors, angiotensine (AT) II receptor blockers, calcium (Ca) channel blockers, statins, fibrates, diuretics, nitrates, H2 receptor blockers, proton pump inhibitors (PPIs), trimetazidine, benzodiazepines, selective serotonine reuptake inhibitors (SSRIs), phosphodiesterase inhibitors (vinpocetine, pentoxyphilline), and nonsteroidal anti-inflammatory drugs (NSAIDs). Exclusion criteria included clopidogrel/dipyridamole use or anticoagulation with warfarin.

4.1.2.5. Statistical analysis:

Data were evaluated as means ± SD (standard deviation) by Student's t-test and the chi squire test. Logistic regression analysis was used to determine the significance of the different parameters as independent risk factors in the development of clopidogrel resistance. The analysis was performed with appropriate adjustments for differences in risk factors and medication usage. For all odds ratios, an exact CI of 95% was constructed in our study. Logistic regression analyses were performed using the statistical package of SPSS 11.0 (SPSS, Chicago, IL, USA).

4.2 Results

4.2.1 Risk profile and previous diseases

The prevalence of hypertension was higher in the group of patients with effective inhibition (80 % vs. 62 %, $p < 0.05$). There was not any other difference in risk profile and previous diseases between the two groups. On the other hand, after the setup of logistic regression analysis between risk profiles and ASA resistance, hypertension did not seem to be an independent predictor

4.2.2 History of medication

In the case of effective platelet aggregation beta-blockers (75 % vs. 55 %, $p < 0.05$) and ACE inhibitors (70 % vs. 50 %, $p < 0.05$), while in the group of ineffective platelet aggregation statins were taken more frequently (52 % vs. 38 %, $p < 0.05$) (see Table 6). After the setup of logistic regression analysis between medications and ASA resistance, only statins remained independent factors of ASA resistance (OR 5.92; 95% CI 1.83 to 16.9; $p < 0.001$).

4.2.3 Hemorheological variables

Patients with effective ASA inhibition had significantly lower plasma fibrinogen level (3,8 g/l vs. 3.3 g/l, $p < 0.05$) and red blood cell aggregation values (28.2 vs. 24.3, $p < 0.01$) (see Table 7).

	Ineffective inhibition	Effective inhibition	p value
hematocrit (%)	41 ± 0.2	42 ± 0.2	n.s
fibrinogen (g/l)	3,8 ± 0.1	3,3 ± 0.1	$p < 0.05$
red blood cell aggregation	28,2 ± 0.3	24,3 ± 0.2	$p < 0.01$
plasma viscosity (mPas)	1,31 ± 0.03	1,3 ± 0.03	n.s
whole blood viscosity at 90 1/s shear rate (mPas)	4,62 ± 0.13	4,58 ± 0.11	n.s

Table 7: *Hemorheological parameters between the study groups.*

(values are represented as mean value ± SEM) .

DISCUSSION

The term of ASA resistance has been used to describe a number of different phenomena, including the inability of ASA to accompanish the followings: 1. to protect individuals from thrombotic complications, 2. to cause a prolongation of bleeding time, 3. to reduce TXA_2 production, or 4. to produce an anticipated effect on one or more in vitro tests of platelet function (57). Approximately one in eight high-risk patients will experience a recurrent atherothrombotic vascular event in the subsequent two years despite taking ASA, while it also fails to prevent 81 % of recurrent serious vascular episodes among high-risk patients. Resistance to this drug is the only one explanation as to why may not be absolutely effective in preventing recurrent vascular events. Other possible reasons include an incorrect diagnosis or noncompliance with the prescribed dosage of medication (58). There are other parameters which play an important role of ASA resistance.

Although several studies examined a set of different factors in ASA resistance by in vitro test of platelet inhibition, in our knowledge this was the first study which analysed the possible role of risk factors, previous diseases medication and hemorheological parameters together.

Poor compliance may be the most important factor of ASA resistance. The higher prevalence of hypertension in the case of effective platelet inhibition may refer this, and it can explain the difference in the prevalence of ACE-inhibitors and beta-blockers. These patients can be tightly controlled by their general practitioners and it can cause better drug-taking compliance. A recent review showed the hazards of discontinuing aspirin. They concluded non-compliance or withdrawal of aspirin treatment has ominous prognostic implication in subjects with or at moderate-to-high risk for CAD. Aspirin discontinuation in such patients should be advocated only when bleeding risk clearly overwhelms that of atherothrombotic events (59).

On the other hand, a recent study showed the potential platelet inhibitory effect of beta-blockers on collagen- and epinephrine-induced platelet aggregation (41), thus an additive effect of these drugs may be involved in the effective antiplatelet therapy. Other publications showed that the benefits of ASA and ACE inhibitors may be attenuated when both agents are used together, although other studies showed no effect of ACE-inhibitors on platelet aggregation (60-62).

Statins are widely used in cardiovascular diseases and their favourable effects were proven by many large-population studies, and they may partially be independent from plasma cholesterol levels, combining lipid-lowering with positive effects on hemorheological conditions and endothelial function (63,64). Statins are also reported to decrease platelet aggregation (65). Recent publications showed the inhibitory effect of statins (especially atorvastatin) on clopidogrel biovability (66,67), but other studies did not confirm this (68,69). Our results suggest that statins may interfere with ASA.

Aspirin, dipyridamole, cilostazol, thienopyridines and glycoprotein IIb/IIIa inhibitors represent the classical examples of the established antiplatelet agents commonly used for the secondary prevention in patients after vascular events. Obviously, the era of expanding antiplatelet regimens and indications may require new agents as the substitutes, or additions to the available strategies. However, recent results of the majority of antiplatelet trials strongly suggest borderline advantages in clinical outcomes, and higher associated bleeding risks with the novel antiplatelet agents or/and regimens. Moreover, unexpected failures, such as lack of efficacy of clopidogrel and aspirin combination for ischemic stroke prevention (MATCH), or use of the same antiplatelet regimen for the primary vascular prevention (CHARISMA) raise legitimate concerns that the concept 'the more the better' may not be valid. Broad use of statins, angiotensin receptor blockers and selective serotonin reuptake inhibitors may be in part responsible for the lack of impressive results with the antiplatelet therapy because each of these drug classes *per se* inhibits platelets (70). Our results showed the possible role of other agents on platelet aggregation and aspirin resistance.

Several studies confirmed that hemorheological parameters are independent risk factors of IHD (45). They play a role in the development of ischemic areas causing insufficient circulation, local stasis, capillary clogging, thrombus formation, slow O_2 transport and cause hypoxia. This is the first study which showed impaired hemorheological parameters as potential background of ASA resistance. When plasma fibrinogen level increases red blood cells adhere and release ADP, which is a potential agonist of platelet aggregation. On the other hand, the aggregated red blood cells migrate in the center of blood flow displacing other cells (platelets) in small vessels, so they can easily contact to the endothelium (46). In the case of effective platelet inhibition we found significantly lower plasma fibrinogen levels compared to the other group.This may be caused by the higher rate of ACE inhibitors, because their favourable effect on hemorheological parameters (especially fibrinogen and plasma viscosity) was shown in a recent study (63). Furthermore, platelets from aspirin-resistant

patients appeared to be more sensitive and activable by ADP. This hypersensitivity could provide a possible explanation for the so-called aspirin resistance, and this could justify therapeutic improvement with alternative antiplatelet agents (71).

A recent meta-analysis showed that patients who are “resistant” to aspirin are at greater risk of clinically important adverse cardiovascular events, regardless of the assay used to measure aspirin resistance. Not only did aspirin resistance have an effect on clinical outcomes but this risk was not ameliorated by currently used adjunct antiplatelet therapies. Patients who were classified as aspirin resistant were at about a fourfold increased risk of non-fatal and fatal cardiovascular, cerebrovascular, or vascular events while taking aspirin than their aspirin sensitive counterparts. This risk can be generalised to a wide variety of patient populations with cardiovascular or cerebrovascular diseases (72). Prospective randomized studies are warranted to elucidate the optimal aspirin dosage for preventing ischemic complications of atherothrombotic diseases.

So, aspirin resistance seemed to be associated with worsening clinical outcome. Our study showed the role of hemorheological parameters in aspirin resistance. We also found antiplatelet effets beyond traditional antiplatelet agents, which may affect the efficacy of aspirin therapy.

Finally, our study has some limitations. The baseline aggregation values of our patients were unknown because at the moment of the examination they already had antiplatelet medication. Their laboratory results were also not measured, which may influence the hemorheological parameters and they may play an important role in the mechanism(s) of resistance.

5. THE POTENTIAL BACKGROUND(S) OF CLOPIDOGREL RESISTANCE

Platelets have a central role in the development of arterial thrombosis and subsequent cardiovascular events. An appreciation of this has made antiplatelet therapy the cornerstone of cardiovascular disease management. Resistance to clopidogrel both in vitro and in vivo has also been described (38). Up to 5-11% of clopidogrel-treated patients were found to be non-responders, while 9-26% were semi-responders (37,38), but the possible mechanisms are still unclear.

5.1 Patients and methods

5.1.1 Patients

157 chronic cardio- and cerebrovascular patients taking 75 mg clopidogrel daily (not combined with aspirin) as antiplatelet agent were involved in our study (83 males, mean age 61 ±11 yrs, 74 females, 63 ±13 yrs).

5.1.2 Methods

5.1.2.1 Platelet aggregation

5.1.2.2. Hemorheological measurements

5.1.2.3 Risk profile and previous diseases

5.1.2.4 History of medications

All these parameters can be seen under 4.1.2 on page 25-27.

5.1.2.5 Measurement of von Willebrand factor

A quantitative direct enzyme immunoassay (Shield Diagnostics Ltd., U.K.) was used for the detection of von Willebrand factor (vWf) activity in human citrated plasma. The wells of the microtitre strips are coated with a preparation of purified monoclonal antibody which recognizes a functional epitope on the vWf antigen. During first incubation the specific antigen in diluted plasma will bind to the antibody coating. The wells are then washed to remove unbounded plasma components. A conjugate of horseradish-peroxidase-labelled mouse anti-human monoclonal anti-vWf conjugate binds to surface-bound antigen in the second incubation. After a further washing step, specifically-bound antibody is traced by the addition of substrate solution. Addition of stop solution terminates the reaction. The amount of conjugate bound is measured in absorbance units. The activity of vWf in unknown sample

can be estimated by interpolation from a dose-response curve prepared from calibrator set according to the 4th International Standard (73).

	ineffective inhibition of platelet aggregation (n = 35)	effective inhibition of platelet aggregation (n = 122)	p value
Mean age (yrs)	62 ± 5	62 ± 6	n.s
Male/female ratio	0.56	0.58	n.s
Mean daily dosage (mg)	75	75	n.s
BMI (kg/m^2)	28.8 ± 0.7	26.1 ± 0.6	0.04
Smoking (%)	27	24	n.s
Diabetes mellitus (%)	36	39	n.s
Hypertension (%)	62	65	n.s
Dyslipidemia (%)	51	49	n.s
IHD (%)	52	51	n.s
AMI (%)	22	21	n.s
Stenosis of the carotid artery (%)	10	11	n.s
TIA (%)	7	7	n.s
Stroke (%)	20	21	n.s
Peripheral arterial disease (%)	8	9	n.s
Deep vein thrombosis (%)	6	7	n.s
Pulmonary embolism (%)	2	3	n.s
β-blockers (%)	56	55	n.s
α-blockers (%)	5	6	n.s
ACE-inhibitors (%)	51	50	n.s
ATII receptor blockers (%)	3	4	n.s
Ca-channel blockers (%)	28	31	n.s
Statins (%)	52	48	n.s
Fibrates (%)	3	3	n.s
Nitrates (%)	25	24	n.s
Diuretics (%)	15	17	n.s
H2 receptor inhibitors (%)	3	4	n.s
PPI (%)	10	9	n.s
Trimetazidine (%)	63	69	n.s
Benzodiazepines (%)	25	10	0.03
SSRIs (%)	28	12	0.04
Phosphodiesterase inhibitors (%)	12	16	n.s

Table 8: *Clinical characteristics of the study groups*

5.1.2.6 Measurement of sP-selectin factor

Determination of the level of sP-selectin was performed by using a sandwich ELISA procedure according to manufacturer's instructions (Bender MedSystems, Austria) using the previously mentioned steps. sP-selectin was determined from platelet poor plasma prepared as described above.

5.1.2.6. Statistical analysis

All these parameters can be seen under 4.1.2 on page 26.

5.2 Results

5.2.1 Platelet aggregation

Among 157 chronic cardio- and cerebrovascular patients involved in our study we found ineffective platelet aggregation in 35 patients (22 %). There was no significant difference in the mean age, the male/female ratio, the dosage of CLP and the duration of therapy between the two groups.

5.2.2 Risk profile and previous diseases

Patients with effective inhibition had lower BMI (28,8 kg/m2 vs. 26,1 kg/m2, $p < 0.05$). There was not any other difference in the prevalence of different risk factors and previous diseases between the two groups (Table 2). After the setup of logistic regression analysis between risk profiles and CLP resistance, BMI remained to be independent factor of CLP resistance (OR 2.62; 95% CI: 1.71 to 3.6; $p < 0.01$) (see Table 8).

5.2.3 History of medication

In the case of ineffective platelet inhibition, benzodiazepines (25 % vs. 10 %, $p < 0.05$) and selective serotonin reuptake inhibitors (28 % vs. 12 %, $p < 0.05$) were taken more frequently (Table 1). After the setup of logistic regression analysis between risk profiles and CLP resistance, they remained to be independent factors of CLP resistance (benzodiazepines: OR 5.83; 95% CI: 2.53 to 7.1; $p < 0.05$ and SSRIs: OR 5.22; 95% CI: 2.46 to 6.83; $p < 0.05$) (see Table 8).

5.2.4 Hemorheological variables and adhesive molecules

There were no significant differences in the hemorheological parameters and in the plasma level of adhesive molecules between the two groups (see Table 9).

	ineffective inhibition	effective inhibition	p value
hematocrit (%)	44 ± 0.2	43 ± 0.3	n.s
fibrinogen (g/l)	3.7 ± 0..4	3.6 ± 0.04	n.s
red blood cell aggregation (%)	29.2 ± 0..22	28.3 ± 0.21	n.s
plasma viscosity (mPas)	1.31 ± 0.01	1.30 ± 0.01	n.s
whole blood viscosity at 90 1/s shear rate (mPas)	4.68 ± 0.03	4.62 ± 0.03	n.s

Table 9. *Hemorheological parameters in the study groups*

(values are represented as mean value ± SEM)

DISCUSSION

Platelet aggregation is a key event in arterial thrombosis. It is also involved in the initiation and development of atherosclerotic lesions, through platelet adhesion to dysfunctional endothelium and release of growth factors and cytokines (19). Adenosine diphosphate (ADP) belongs to the key mediators of platelet stimulation and mediates its effect through two 7 transmembrane receptors, P2Y1 and P2Y12 (32). P2Y12 plays a particularly important role in platelet aggregation, since its coupling to a GI protein is responsible both for stabilizing platelet aggregates and for amplifying aggregation induced by ADP and other agonists (66). The importance of the ADP receptor P2Y12 is emphasized by the fact that patients with cardiovascular diseases derive a greater benefit when platelet aggregation is blocked by thienopyridines than when platelet function is inhibited by aspirin (33). Clopidogrel-induced platelet inhibition is patient-specific (37,38). The concept of clopidogrel resistance has emerged in the medical literature to reflect the failure to inhibit platelet function in vitro, although its existence and definition remain to be established. It has been proposed that the term resistance encompasses patients for whom clopidogrel does not achieve its pharmacological effect, and failure of therapy reflects patients who have recurrent events on therapy (37). The prevalence of clopidogrel nonresponse in patients is evaluated between 4% and 30% 24 h after administration (37,38). The reported rates vary between studies because of the technique used to measure the extent of platelet aggregation and the presence of factors contributing to greater baseline platelet reactivity. Furthermore, the definition of nonresponders is not standardized. We defined clopidogrel resistance as an inadequate decrease of the aggregation index induced by the appropriate agonists compared to healthy volunteers. We found that higher BMI is an independent factor of clopidogrel resistance, which is in concordance with the results of Angiolillo et al. (74,75). High dose of clopidogrel (600 mg loading dose) was shown to be more effective compared with 300 mg (76). Our results suggest that long term treatment should be weight-adjusted. There were no other differences in cardiovascular risk profile or previous disease history between the two groups, although smoking might play an important role of clopidogrel metabolism by the way of P 450 1A2 (38). Many drugs have an increasing effect of platelet function (77), thus they may be associated with platelet inhibition. Certain statins were reported to may decrease the efficacy of clopidogrel treatment by the way of CYP3A4 (37,38). In our study statins did not interfere with the efficacy of clopidogrel, but SSRIs and benzodiazepines did. Recent studies

showed that benzodiazepines, especially alprazolam, triazolam and midazolam are metabolised and eliminated by CYP3As. It is also shown that certain SSRIs, as fluvoxamine are potent inhibitors of CYP1A2 and nefazodone is a potent inhibitor of CYP3A4, which isoenzymes play an important role in clopidogrel metabolism (78-80). CYP3A4 activity plays a role in clopidogrel resistance and metabolic interactions on this isoenzyme may decrease the metabolism and antiplatelet effect of clopidogrel (81). On the other hand, diazepam and clonazepam in a concentration-dependent manner inhibited thrombin, ADP or AA-stimulated platelet aggregation and the thrombin-induced increase in free intracellular Ca^{2+} (82). Thus receptorial interactions may also play a role in this phenomenon. The severity of atherosclerosis may be associated with resistance to antiplatelet therapy in patients treated with aspirin (37). The level of hemorheological parameters, von Willebrand factor and P-selectin were shown to be correlated with the progression of atherosclerosis (82). Our findings suggest that clopidogrel resistance may not be associated with the progression of atherosclerosis.

A recent systematic review on prevalence and clinical consequences of laboratory-defined clopidogrel nonresponsiveness among patients undergoing PCI indicates that in approximately 1 in 5 of them, clopidogrel nonresponsiveness can be found and that this condition appears to be related to worsened clinical outcomes. Our results indicate that use of a 600-mg clopidogrel loading dose in patients undergoing PCI may result in a more rapid and stronger antiplatelet effect, which needs to be confirmed in large prospective studies. Future studies are also warranted to examine which method and time of determining clopidogrel nonresponsiveness could be used in clinical practice to identify patients at the highest risk. Furthermore, there is a clear need for future studies addressing alternative strategies for these high risk patients (83).

A recent study suggested that adjusting clopidogrel loading dose according to platelet reactivity measured by VASP index is safe and may significantly improve clinical outcome after PCI in patients with clopidogrel resistance. Moreover, the very high sensitivity of the VASP index at a cutoff value of 50% may be considered of clinical interest in avoiding subacute stent thrombosis and may justify routine monitoring for clopidogrel resistance in high-risk population subsets (84).

In summary, our study is among the first studies which examined the potential background(s) of clopidogrel resistance. Clopidogrel resistance is a clinical entity with serious outcome. Monitoring and adjusting the antiplatelet therapy may be associated with decreased recurrent vascular events. We showed that BMI is associated with low response to clopidogrel and psychotropic agents (benzodiazepines and SSRIS) but not statins may inhibit the metabolism of clopidogrel possibly by the way of CYP3A4 and CYP1A2. Finally, our study had some limitations. The baseline aggregation values of our patients were unknown because at the moment of the examination they already were on antiplatelet medication. Their blood chemistry was also not measured, and these parameters might influence the hemorheological parameters (especially hyperlipidemia) and they may play a role in the mechanism of resistance. Finally, we did not examine the patients directly, their data (BMI, high blood pressure, etc.) were extracted from hospital case notes.

6. SUMMARY

Although we have the data of several large trials, the role of hemorheological parameters and their possible relation to age is still under-represented in circulation research and in the clinical practice. „Classic" risk factors can also influence hemorheological parameters. A positive correlation between BMI and blood viscosity and its determinants has been demonstrated in several studies. Arterial hypertension is in association with increased blood viscosity. Smoking increases in a reversible way plasma and whole blood viscosity, partly by increasing hematocrit and fibrinogen. Many drugs have potential effect on hemorheological parameters. Relatively few study examined the associations between hemorheological parameters and increasing age. They reported a not very pronounced increase and these reports depended on the populations studied and are controversial. To evaluate the effect of risk profile, previous diseases and medication, 623 patients were selected from the examined group with the same parameters. Our results suggest, that in a homogenous population, hemorheological parameters are independent of aging, thereby altered hemorheological parameters are much more associated with different diseases and the severity of atherosclerosis, than with aging alone.

Our study investigating the efficacy of routine antiplatelet medication confirmed the existence of both aspirin and thienopyridine non-responder individuals in the general population. Many different ways to perform aggregometry have been published. All tests have in common that their widespread clinical use is substantially limited due to complex preanalytic factors, reduced specificity and reproducibility. The results of these tests may become more comparable after the standardization of the different methods.

There is no debate that long term antiplatelet use attenuates the risks of myocardial infarction, stroke, and vascular related deaths in patients with cardiovascular diseases, but a significant number of patients prescribed antiplatelet as antithrombotic therapy have major adverse vascular related events each year. The major controversy about antiplatelet therapy is why particular patients do not benefit from such therapy and how they might be identified. It has been suggested that some patients require a higher dose of antiplatelet than is normally recommended to achieve the expected antiplatelet effect - for example, inhibition of platelet function or inhibition of platelet thromboxane A2 synthesis. It is unclear whether these patients simply receive too low antiplatelet dose, are not compliant, have differing abilities to absorb antiplatelet, or have an underlying genetic disposition that renders antiplatelet

ineffective. Such patients have been labelled antiplatelet “resistant” - that is, their platelets are not affected in the same way or are affected differently from the platelets of those who seem to benefit from antiplatelet therapy (antiplatelet “sensitive” patients with no subsequent adverse cardiovascular event). Little consistency exists about which measure should be used to identify patients who seem resistant to antiplatelet. Also, few studies have assessed the effect of antiplatelet resistance on clinically important outcomes.

A recent systematic review on prevalence and clinical consequences of laboratory-defined clopidogrel nonresponsiveness among patients undergoing PCI indicates that in approximately 1 in 5 of them, clopidogrel nonresponsiveness can be found and that this condition appears to be related to worsened clinical outcomes. Our results indicate that use of a 600-mg clopidogrel loading dose in patients undergoing PCI may result in a more rapid and stronger antiplatelet effect, which needs to be confirmed in large prospective studies. Future studies are also warranted to examine which method and time of determining clopidogrel nonresponsiveness could be used in clinical practice to identify patients at the highest risk. Furthermore, there is a clear need for future studies addressing alternative strategies for these high risk patients.

A recent meta-analysis showed that patients who are “resistant” to aspirin are at greater risk of clinically important adverse cardiovascular events, regardless of the assay used to measure aspirin resistance. Not only did aspirin resistance have an effect on clinical outcome but this risk was not ameliorated by currently used adjunct antiplatelet therapies. Patients who were classified as aspirin resistant were at about a fourfold increased risk of non-fatal and fatal cardiovascular, cerebrovascular, or vascular events while taking aspirin than their aspirin sensitive counterparts. This risk can be generalised to a wide variety of patient populations with cardiovascular or cerebrovascular disease. Prospective randomized studies are warranted to elucidate the optimal aspirin dosage for preventing ischemic complications of atherothrombotic disease.

The mechanisms underlying antiplatelet resistance can be multifactorial. Impaired hemorheological parameters (especially plasma fibrinogen) seemed to be associated only with aspirin, but not with clopidogrel resistance. When plasma fibrinogen level increases red blood cells adhere and release ADP, which is a potential agonist of platelet aggregation. On the other hand, the aggregated red blood cells migrate in the center of blood flow displacing other cells (platelets) in small vessels, so they can easily contact to the endothelium, releasing ADP, which is a strong agonist of platelet activation.

In the case of effective platelet inhibition we found significantly lower plasma fibrinogen levels compared to the other group.

Increasing BMI was associated with lower response to clopidogrel therapy. We found that higher BMI is an independent factor of clopidogrel resistance, which is in concordance with the results of previous studies. Our results suggest that long term treatment should be weight-adjusted.

Statins remained an independent predictor of aspirin resistance and benzodiazepines and SSRIs remained an independent predictor of clopidogrel resistance even after adjustment for risk factors and medication use. The COX-1 enzyme dependent antiplatelet effect of statins has been previously showed. They may interfere with the COX-1 inhibitory effect of aspirin. Recent studies showed that benzodiazepines, especially alprazolam, triazolam and midazolam are metabolised and eliminated by CYP3As. It is also shown that certain SSRIs, as fluvoxamine are potent inhibitors of CYP1A2 and nefazodone is a potent inhibitor of CYP3A4, which isoenzymes play an important role in clopidogrel metabolism. CYP3A4 activity plays a role in clopidogrel resistance and metabolic interactions on this isoenzyme may decrease the metabolism and antiplatelet effect of clopidogrel. Our result showed the potential antiplatelet effect of different (not antiplatelet) agents, which may affect the efficacy of routine antiplatelet therapy.

Finally, despite of recent clinical trials and meta-analysises, the definition of antiplatelet resistance is still unclear. The role of other parameters (especially the role of drug interactions) is poorly studied. Large, population based, randomized studies are needed to clarify the previous results.

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